Enhanced chemosensitivity of drug-resistant osteosarcoma cells by lentivirus-mediated *Bcl-2* silencing

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ABSTRACT

The *Bcl-2* gene is frequently overexpressed in malignancy and is responsible for the resistance induced by chemotherapeutic drugs. The aim of this study was to investigate whether the inhibition of *Bcl-2* by lentivirus-mediated RNA interference would enhance doxorubicin cytotoxicity in the drug-resistant human osteosarcoma MG63 cells. Downregulation of *Bcl-2* was confirmed by quantitative reverse transcription PCR and Western blotting. Moreover, the ratio of Bcl-2/Bax decreased due to the downregulation of Bcl-2 expression and the upregulation of Bax expression. Decreased cyclin D1 expression was also detected. Flow cytometry and MTT assays revealed that *Bcl-2* knock-down increased cellular apoptosis and the MG63 cells became sensitive to doxorubicin. However, no detectable alterations in MDR1 or Bcl-xl expression were observed. Therefore, lentivirus-mediated *Bcl-2* knock-down may sensitize these human osteosarcoma cells to doxorubicin and provide a potential therapeutic strategy for osteosarcoma.

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Introduction

Osteosarcoma is the most common primary malignant bone tumor affecting children and adolescents. The application of neoadjuvant chemotherapy and surgery improved the five year survival rate to approximately 70% by the mid-1980s [1]. However, despite further improvements in osteosarcoma therapy over the last 20 years, the overall survival of patients has reached a plateau [2]. A poor response to chemotherapy for osteosarcoma is associated with a poorer outcome and a lower survival rate [3]. Most chemotherapeutic agents, as well as radiation, induce cancer cell death by multiple apoptotic pathways. However, increased drug efflux and decreased drug influx, drug inactivation, alterations in drug targets, increased DNA damage repair and evasion of apoptosis have all been demonstrated to contribute to intrinsic or acquired tumor chemoresistance [4]. As in other diseases, recent advances in the understanding of the molecular mechanisms of osteosarcoma have provided the basis for novel gene therapies *in vitro* and *in vivo*; thus, the inhibition of mutated key oncogenes may be an effective therapeutic strategy in osteosarcoma [5,6].

The B-cell CLL/lymphoma 2 (Bcl-2) family of proteins includes a number of apoptotic regulators with opposing functions. Bcl-2, a well characterized oncoprotein, mediates an anti-apoptotic function principally by inhibiting the release of mitochondrial cytochrome *c* into the cytoplasm, thereby blocking Apaf1-mediated caspase-9 activation. It therefore contributes to tumorigenesis and decreased efficacy of chemotherapy in various malignancies by inhibition of apoptosis [7]. Bcl-2 is expressed at a high level in osteosarcoma [8,9]. Therefore, elevated expression of Bcl-2 may contribute to the intrinsic chemoresistance of osteosarcoma, and may be a promising target for gene therapy for this cancer. However, the role of anti-apoptotic Bcl-2 on osteosarcoma resistance has not been sufficiently investigated.

Bcl-2-associated X protein (Bax) is a pro-apoptotic member of the Bcl-2 family, and the activation of the *Bax* gene increases sensitivity to apoptosis in osteosarcoma cells [10]. The decreased Bcl-2/Bax ratio is due to the induction of apoptosis and the suppression of cell growth in many tumors including osteosarcoma [11,12].

Cyclin D1 acts as a key regulator of cell proliferation. It plays a crucial role in the regulation of the G1 to S phase progression and in tumorigenesis. Bcl-2 overexpression was found to induce cyclin D1 expression in the human breast epithelial cell line MCF 10A [13]. Bcl-2 silencing in mantle cell lymphoma induces a decrease in cyclin D1 [14]. Therefore, we have focused on the possible influence of Bcl-2 on cyclin D1 in osteosarcoma.

As discussed above, we supposed that Bcl-2 blockage may decrease the ratio of Bcl-2/Bax, thereby interrupting tumorigenesis and enhancing drug cytotoxicity in osteosarcoma cells. To test our hypothesis, we performed a lentivirus-mediated RNA interference (RNAi) study, targeting *Bcl-2* in human osteosarcoma cells. DXR cytotoxicity and cell apoptosis were investigated *in vitro* by MTT assays and flow cytometry. The expression of Bcl-2, Bax and cyclin D1 were detected by quantitative reverse transcription PCR (Q-PCR) and Western blotting.

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Materials and methods

Cell lines and culture conditions. The human osteosarcoma MG63 cell line (American Type Culture Collection, ATCC, No. CRL_1427) was cultured in Dulbecco's modified Eagle's medium (Advanced DMEM, Gibco Cat. 12491-015, California, USA) supplemented with 10% fetal bovine serum (Gibco). The drug-resistant osteosarcoma cell line MG63/DXR, which was kindly provided by Dr. Yoshio Oda [15], was selected in a step-wise manner by exposing drug-sensitive MG63 cells to increasing doses of doxorubicin (DXR), and was maintained in the conditioned medium plus 1 μg/ml DXR(Sigma–Aldrich, Cat. No. D1515, Missouri, USA).

Small interfering RNA (siRNA) design, lentivirus construction and production. The siRNA sequences (Table 1) targeting the human *Bcl-2* transcript (GenBank ID: NM_000633) were designed using the BLOCK-iT RNAi Designer algorithm (http://rnaidesigner.invit-rogen.com/rnaiexpress/) and we avoided unintentional silencing of non-target host cell genes by checking for homology with a Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) search. A scrambled-sequence oligonucleotide was cloned for a negative control shRNA. Pairs of complementary oligonucleotides of these sequences were synthesized (Invitrogen, Shanghai, China), annealed and cloned into the pshRNA-H1-GFP lentivector (System Biosciences, California, USA; GFP: green fluorescent protein) at a site 3' to the human H1 promoter, resulting in the lentivirus plasmids LV-*Bcl-2* siRNAs 1–4 (Table 1). MG63 cells were infected with one of the four siRNAs using Lipofect-amine™ 2000 (Invitrogen, Cat: 11668-027, California, USA). After 48 h, *Bcl-2* expression was detected. *Bcl-2* siRNA 2 best suppressed *Bcl-2* expression, and was therefore selected for further experiments (data not shown). To produce lentivirus, 293TN producer cells (System Biosciences, Cat. #LV900A-1) were co-transfected with the pPACK Packaging Plasmid Mix (System Biosciences) and the pshRNA-H1-GFP lentivector containing the siRNA sequences or GFP plasmid, using Lipofectamine™ 2000 (Invitrogen). Lentiviral supernatants were harvested after 48 h of incubation and filtered through 0.45 μm Polyvinylidene Fluoride (PVDF, Millipore, Cat. #IPVH00010, Massachusetts, USA) membrane. The titers were determined and modulated to 1×10^4 infectious unit/μl with PBS. The highest infection efficiency, as determined by fluorescence microscopy, was obtained at a multiplicity of infection (MOI) of 15, and minimal apoptosis was detected by flow cytometry examination.

Stable infection of MG63 and MG63/DXR cells. There were four experimental groups: MG63 cells, MG63/DXR cells not infected with lentivirus, MG63/DXR cells infected with LV-negative at 15 MOI and MG63/DXR cells infected with LV-*Bcl-2* siRNA 2 at 15 MOI. Infections to MG63 and MG63/DXR cells were performed according to the manufacturer's instructions (System Biosciences). Ninety-six hours after infection, fluorescent expression was identi-fied in more than 90% of cells. Culture of the brightest fluorescent cells was scaled-up for further experiments.

Q-PCR analysis. DNA-free total RNA was extracted using TRIZOL (Invitrogen, Cat: 15596-026). Q-PCR was performed in triplicate using a SYBR PrimeScript™ RT-PCR Kit II (Takara, DRR083A, Otsu, Japan) in a Thermal Cycler Dice™ Real Time System (Takara, TP600). The amplification program for all primer sets involved 40 cycles of 95 °C denaturation for 10 s, 60 °C annealing for 20 s and 72 °C extension for 20 s. The relative expression of Bcl-2, Bax and cyclin D1 mRNA was calculated by the comparative Ct method [16]. The relative expression of Bcl-xl and MDR1 before and after doxorubicin treatment (1.5 μg/ml for 48 h) was also calculated by the same method. β-actin mRNA levels were used for normalization of RNA expression. All experiments were repeated at least three times.

Western blotting. Cell lysates were separated on 10% SDS–PAGE gels and transferred onto PVDF membranes. Proteins were probed overnight at 4 °C with one of the following primary antibodies: Bcl-2 (50E3) rabbit mAb (1:1000,Cell Signaling Technology, CST, Cat. #2870, Massachusetts, USA), Bax antibody (1:1000, CST, Cat. #2774), Bcl-xl (pS62) rabbit antibody (1:1000, Millipore, Cat. #AB3573, Massachusetts, USA), cyclin D1 (DCS6) mouse mAb (1:2000, CST, Cat. #2926), Caspase-3, large subunit & perform, Rabbit mAb (1:1000, Millipore, Cat. #AB1899), MDR1 rabbit antibody (1:1000, Sigma, Cat. #HPA002199, Missouri, USA) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) rabbit mAb (1:1000, CST, Cat. #2118). The membrane was then incubated with their respective horseradish peroxidase-conjugated secondary antibodies (1:1000–1:2000, CST,Cat. #7074, #7076). Following this, signals were detected in triplicate by ECL Western blotting KIT (Thermo Scientific, 32209, Illinois, USA), using GAPDH as the internal standard.

MTT assay. For cell proliferation measurement, with the treatment of DXR (1.5 μg/ml), the number of viable cells in the four groups was determined by MTT assay at daily intervals (0, 24, 48, 72 h). Fifteen microliters of MTT (5 mg/ml) was added to each well. After 4 h, the supernatant was removed and replaced with 100 μl dimethyl sulfoxide (DMSO). The dye crystals were dissolved and the absorbance was measured at 570 nm. For DXR cytotoxicity measurement, cells were exposed to DXR for 48 h from 1.0 μg/ml to 10.0 μg/ml according to concentration gradient, Then MTT was added, followed by DMSO. The absorbance was also measured at 570 nm. The 50% inhibitory concentration (IC_{50}) were calculated. Each experiment was performed in triplicate and repeated three times.

Flow cytometry. For determination of the cell cycle, cells were rinsed twice in PBS and fixed with ice-cold 70% ethanol. 100 μl of Ribonuclease inhibitor (RNase I, 1 mg/ml) and 100 μl of Propidium iodide (PI, 400 μg/ml) were added to the cell suspensions, incubated at 37 °C for 30 min and analyzed by flow cytometry. For

Table 1
Human *Bcl-2* target sequences and oligonucleotide sequences (scrambled siRNA as the negative).

siRNA oligonucleotide	Target sequence (5′–3′)	Oligonucleotide sequences
siRNA1	GCTGCACCTGACGCCCTTC	Forward:5′-GATCCGCTGCACCTGACGCCCTTCCTTCCTGTCAGAGAAGGGCGTCAGGTGCAGCTTTTTG -3′
		Reverse:5′-AATTCAAAAAGCTGCACCTGACGCCCTTCTCTGACAGGAAGGAAGGGCGTCAGGTGCAGCG -3
siRNA2	GCTGGATTATAACTCCTCT	Forward:5′-GATCCGCTGGATTATAACTCCTCTCTTCCTGTCAGAAGAGGAGTTATAATCCAGCTTTTTG -3′
		Reverse:5′-AATTCAAAAAGCTGGATTATAACTCCTCTTCTGACAGGAAGAGAGGAGTTATAATCCAGCG -3
siRNA3	GGATGACTGAGTACCTGAA	Forward:5′-GATCCGGATGACTGAGTACCTGAACTTCCTGTCAGATTCAGGTACTCAGTCATCCTTTTTG -3′
		Reverse:5′-AATTCAAAAAGGATGACTGAGTACCTGAATCTGACAGGAAGTTCAGGTACTCAGTCATCCG -3
siRNA4	GGAGAACAGGGTACGATAA	Forward:5′-GATCCGGAGAACAGGGTACGATAACTTCCTGTCAGATTATCGTACCCTGTTCTCCTTTTTG -3′
		Reverse:5′-AATTCAAAAAGGAGAACAGGGTACGATAATCTGACAGGAAGTTATCGTACCCTGTTCTCCG -3
siRNA-negative	CGTTTAACTCTCCCAACCA	Forward:5′-GATCCCGTTTAACTCTCCCAACCACTTCCTGTCAGATGGTTGGGAGAGTTAAACGTTTTTG -3′
		Reverse:5′-AATTCAAAAACGTTTAACTCTCCCAACCATCTGACAGGAAGTGGTTGGGAGAGTTAAACG -3

A series of siRNAs directed against the human *Bcl-2* mRNA sequence were designed to correspond to different regions of the transcript. Target sequences of the siRNA oligonucleotides and their corresponding positions in the human *Bcl-2* transcript are shown.

detection of apoptosis, the cells were harvested after pre-treatment with DXR for 48 h, rinsed twice with PBS and resuspended, then stained with an Annexin V-FITC and PI Apoptosis kit (BD Biosciences, Cat. 556570, California, USA). Finally, cell apoptosis was analyzed by BD FACSCanto II flow cytometry system (BD Biosciences). Experiments were conducted in at least triplicate.

Statistical analysis. Statistical analyses were performed using the Student's *t*-test with SPSS 11.0(SPSS Inc. Chicago) statistical software. $p < 0.05$ was considered statistically significant.

Results

Generation of stable cell lines expressing LV-Bcl-2 shRNA

Ninety six hours after infection, fluorescent expression was identified in more than 90% of MG63/DXR cells. This demonstrates the high infection efficiency of the lentiviral vector.

Lentivirus-mediated RNAi silencing specifically inhibits the expression of Bcl-2 mRNA and protein; however, this is associated with elevated Bax and decreased cyclin D1 in the MG63/DXR cell line

The mRNA and protein levels of Bcl-2, Bax and cyclin D1 in each group were measured by Q-PCR and Western blotting (Fig. 1). The mRNA and protein levels of Bcl-2 and cyclin D1 in the MG63/DXR cells were both higher than in the parental MG63 cells (Fig. 1A, B and D), which suggested that constitutive exposure to DXR upregulates Bcl-2 and cyclin D1 levels. When compared with uninfected MG63/DXR cells, the mRNA and protein levels of Bcl-2 in siRNA-infected MG63/DXR cells were significantly reduced, by 68.9% and 72.0%, respectively (Fig. 1A and D). Interestingly, when *Bcl-2* silencing was induced, Bax expression increased (Fig. 1D, data for mRNA not shown). This resulted in a significantly decreased Bcl-2/Bax ratio in the *Bcl-2*-silenced cells ($p < 0.01$) (Fig. 1C). The mRNA and protein levels of cyclin D1 were also decreased in the *Bcl-2*-silenced cells (Fig. 1B and D). We found a similar level of pro-caspase-3 before *Bcl-2* silencing, thereafter significant downregulation of cleaved

caspase-3 protein was detected in MG63/DXR and MG63/DXR LV-negative cells compared with MG63 cells ($p < 0.01$), but not in the *Bcl-2*-silenced cells.

Lentivirus-mediated Bcl-2 silencing inhibited proliferation of MG63 cells

We analyzed the cell proliferation of the parental, control and infected cells using MTT assays. On day 4, the proliferation of MG63/DXR cells was accelerated 1.53-fold relative to the parental MG63 cells ($p < 0.05$) (Fig. 2). However, on the same day, the proliferation of MG63/DXR Bcl-2-silenced cells was significantly reduced (0.55-fold proliferation relative to uninfected MG63/DXR cells; $p < 0.01$).

mRNA and protein levels are stable with Bcl-xl and MDR1 while the RNA silencing

As shown in Fig. 3, the Bcl-xl and MDR1 mRNA and protein levels were detected before and after the infection and DXR treatment (1.5 μg/ml, 24 h). There was no difference in Bcl-xl levels between these groups ($p > 0.05$, Fig. 3A and C). MG63 cells expressed a slight level of MDR1, like the other groups, it kept a stable level after the DXR treatment ($p > 0.05$, Fig. 3B and C), MG63/DXR, MG63/DXR Lv-negative and MG63/DXR *Bcl-2*-silenced cells all expressed a upregulated level of MDR1($p < 0.01$), but no statistical difference within these three cell lines ($p > 0.05$).

Changes in sensitivity to DXR in MG63/DXR cells after lentiviral-mediated RNA interference

We investigated whether *Bcl-2* downregulation would influence the sensitivity of drug-resistant MG63/DXR cells to DXR (Fig. 4). The IC$_{50}$ of DXR treatment was greatly increased to 8.507 μg/ml, in comparison with the 0.936 μg/ml of the parental MG63 cells. The IC$_{50}$ of *Bcl-2* siRNA-treated MG63/DXR cells was decreased significantly to 1.013 μg/ml ($p < 0.01$, Fig. 4A). Flow cytometry analysis showed that after DXR treatment for 48 h, the proportion of
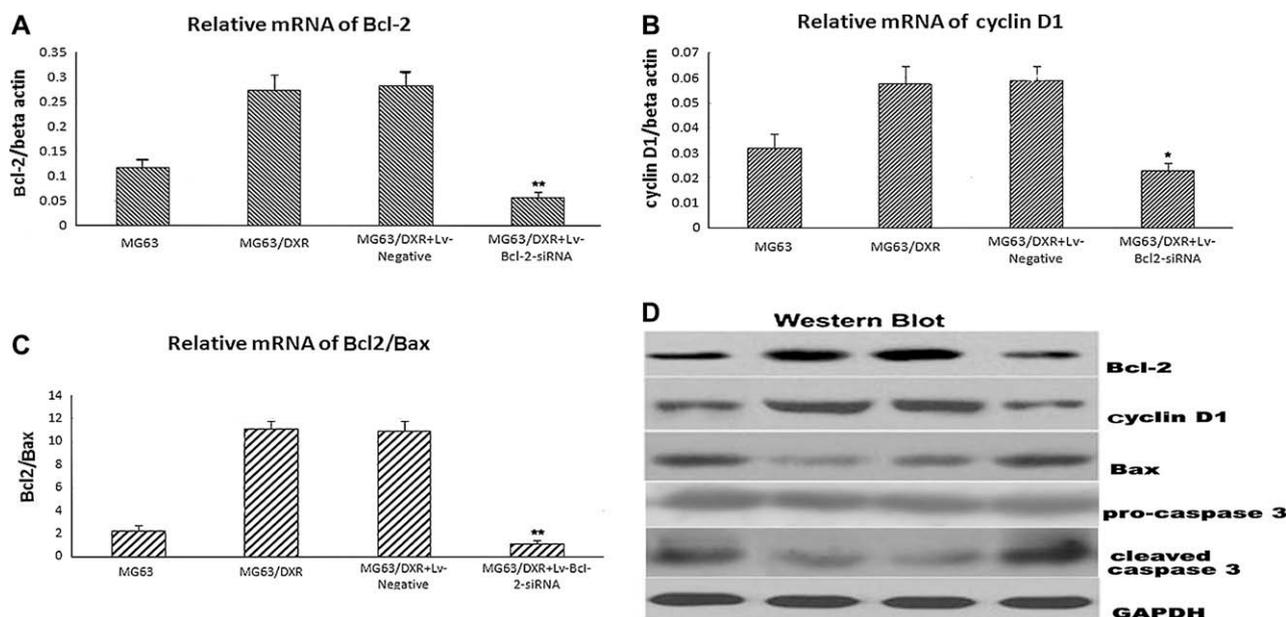

Fig. 1. Decreased Bcl-2, cyclin D1 and Bcl-2/Bax ratio. Compared with MG63 cells, the mRNA and protein levels of Bcl-2 and cyclin D1 were elevated in the drug-resistant MG63/DXR cells, but the elevation was removed in the *Bcl-2*-silenced MG63/DXR cells ($p < 0.05$). (A–C) Relative mRNA levels of Bcl-2, cyclin D1 and the Bcl-2/Bax ratio, respectively. (D) Bax expression was decreased in the MG63/DXR cells, but was upregulated in the *Bcl-2*-silenced cells ($p < 0.05$). Cleaved caspase-3 protein increased in MG63 and *Bcl-2*-silenced MG63/DXR cells, whereas pro-caspase-3 showed no statsistical difference in the four groups ($p > 0.05$).
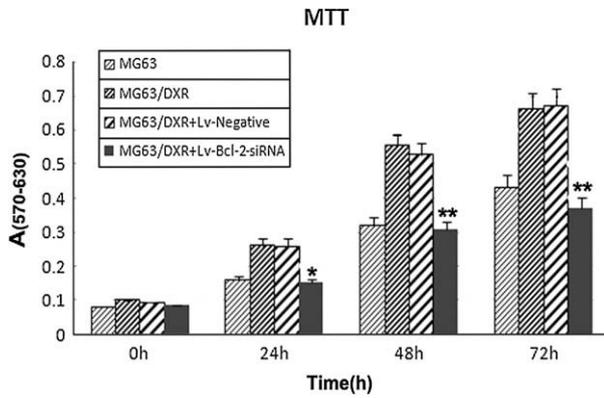
MTT

Fig. 2. Inhibition of proliferation by *Bcl-2* silencing. With the treatment of DXR (1.5 μg/ml), cell proliferation was measured at daily intervals (0, 24, 48, 72 h) by absorbance (A) in the MTT assay. Drug-resistant MG63/DXR cells grew in an accelerated manner (there were 1.53-fold cells than the parental MG63 cells on day 4). However, the proliferation of *Bcl-2* siRNA-infected MG63/DXR cells was significantly decreased ($^*p < 0.05$, $^{**}p < 0.01$).

apoptotic cells was elevated significantly in *Bcl-2*-silenced MG63 cells (77.29%) in comparison with uninfected MG63/DXR cells (19.49%, $p < 0.01$, Fig. 4B).

The effects of downregulation of Bcl-2 expression on the cell cycle

In cell cycle analysis, there was a dramatic increase in the sub-G1 population after PI staining in the *Bcl-2*-silenced MG63/DXR cells (75.23%) compared with the parental (43.36%) and uninfected MG63/DXR cells (32.75%) (Fig. 4C).

Discussion

In the present study, we have demonstrated that chronic exposure to DXR induces upregulation of the oncogenic Bcl-2 and cyclin D1 in human osteosarcoma MG63 cells. We have established MG63 cell lines in which *Bcl-2* was stably knocked down by lentiviral vector-mediated RNAi. Then we demonstrated that the cytotoxicity of DXR was enhanced by silencing *Bcl-2* in drug-resistant human osteosarcoma MG63 cells, the chemoresistance of which was induced by multiple factors, including the acquired overexpression of P-glycoprotein. We also detected elevated Bax and decreased cyclin D1 in the *Bcl-2*-silenced MG63/DXR cells by measuring mRNA and protein expression. Furthermore, caspases-3, Bcl-xl, MDR1 expression were also determined.

Bcl-2 has been regarded as a potential molecular target for gene therapy on the basis of its ability to disrupt the apoptotic cascade and to confer resistance to chemotherapy and radiotherapy in tumors [7,17,18]. Previous reports indicate that Bcl-2 confers the intrinsic resistance of osteosarcoma to drug-induced apoptosis [19,20]. With chemotherapy as a selective pressure, cancer cells do increase their acquired resistance to apoptosis, most often by upregulation of oncogenes in the upstream apoptotic pathway [21]. DXR could activate the Bcl-2 pathway, and the upregulated Bcl-2 may contribute to drug resistance, which may be an adaptation of malignant cells to constitutive chemotherapeutic stress [22]. In the current study, we have studied a drug-resistant human osteosarcoma MG63/DXR cell line [15]. A main mechanism of the drug resistance in MG63/DXR cells is associated with the upregulation of multidrug-resistance-1(MDR1)/P-glycoprotein after constitutive exposure to the topoisomerase-2 inhibitor DXR. We have detected higher levels of Bcl-2 and cyclin D1 expression in the multi-drug-resistant MG63/DXR cell line in comparison with the
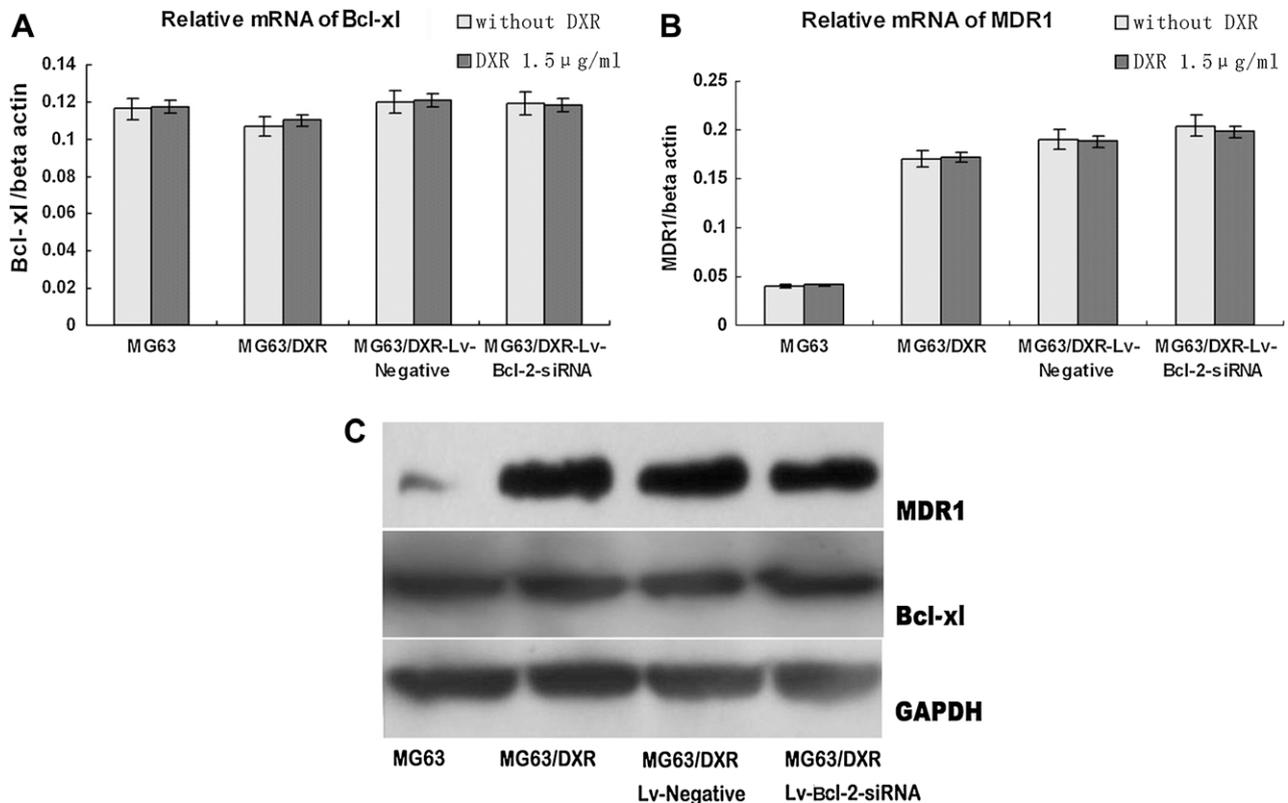

Fig. 3. Stable levels of Bcl-xl and MDR1. The mRNA and protein levels were stable with Bcl-xl and MDR1 while RNA silencing were performed. (A) There was no difference of Bcl-xl mRNA levels within these groups. (B) MG63/DXR, MG63/DXR Lv-negative and MG63/DXR *Bcl-2*-silenced cells all expressed a significantly upregulated level of MDR1 mRNA in comparison with the parent MG63 cells ($p < 0.01$), (C) Western blotting showed a similar result of Bcl-xl and MDR1 expression in four groups.
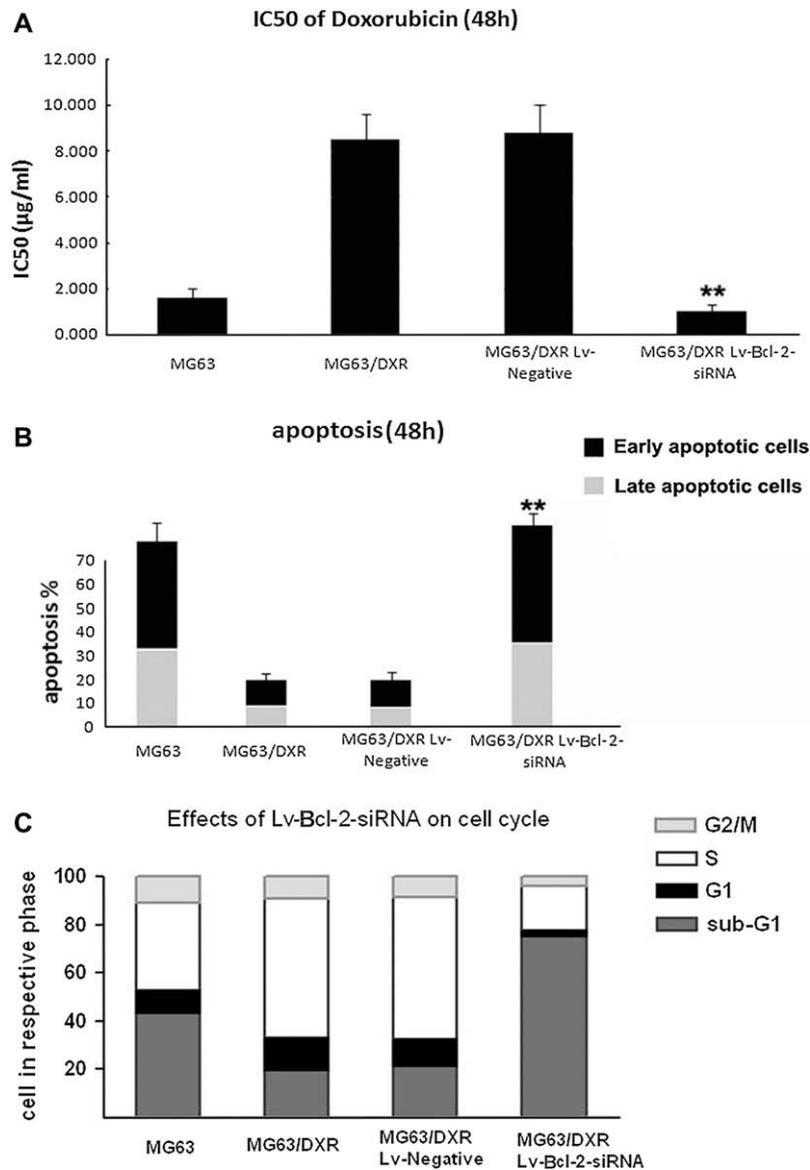
Fig. 4. Enhanced apoptosis and a prolonged cell cycle induced by *Bcl-2* silencing (A) The IC_{50} of the MG63/DXR cells was augmented to 8.507 µg/ml compared with 0.936 µg/ml in the parental MG63 cells, but the IC_{50} of *Bcl-2* siRNA cells was significantly decreased to 1.013 µg/ml (** $p < 0.01$). (B) 77.29% of cells were apoptotic in *Bcl-2*-silenced MG63/DXR cells, in comparison with 19.49% in uninfected MG63/DXR cells (** $p < 0.01$). (C) There was a dramatic increase in the sub-G1 population in the *Bcl-2*-silenced MG63/DXR cells (75.23%) compared with the parental (43.36%) and uninfected (21.21%) MG63/DXR cells.

parental MG63 cells. Therefore, the upregulation of oncogenic Bcl-2 and cyclin D1 in the MG63/DXR cell line may also contribute to its acquired chemoresistance, besides the MDR1-mediated mechanism. All of the above indicate that Bcl-2 could be a potential target of molecular therapy in osteosarcoma.

Our study is the first report describing an shRNA-mediated *Bcl-2* silencing strategy to enhance drug cytotoxicity in osteosarcoma. Previous shRNA-based therapeutic strategies have inhibited *Bcl-2* with exquisite selectivity, and also showed features superior to using antibodies or inhibitors, partly because shRNAs are easily applied to the target [23]. We applied a lentiviral delivery system to infect cells. Lentivirus-mediated infection is invariably more efficient than other methods due to the ability of lentivirus to infect both dividing and non-dividing cells homeochronously, resulting in almost 100% efficiency of the transgene. Moreover, RNAi mediated by lentiviral vectors allows for a prolonged reduction in expression of the targeted gene [24]. Our Q-PCR and Western blot assays revealed that expression of *Bcl-2* in the infected cell line was markedly decreased, which demonstrated that the lentivirus-medi-

ated RNAi technique is an effective way to modulate *Bcl-2* expression in the drug-resistant MG63 cell line.

Our study showed that silencing *Bcl-2* induced the upregulation of Bax. This is in accordance with previous reports showing that the expression of Bax was negatively regulated by Bcl-2 [25]. We demonstrated that the resultant reduction in the Bcl-2/Bax ratio, which is associated with the induction of apoptosis and suppression of cell growth in many tumors, also resulted in a decreased IC_{50} and an increased apoptotic proportion in MG63/DXR *Bcl-2*-silenced cells, thus plays a key pro-apoptotic role in human osteosarcoma. Caspase-3 that mediates the intrinsic apoptotic pathway was investigated in the study. We consider the slightly expressed cleaved caspase-3 in MG63/DXR and MG63/DXR LV-negative cells results from the elevated Bcl-2 and is associated with the resistance to DXR-induced apoptosis, while *Bcl-2* silencing contribute to the activation of caspase-3. Furthermore, we detected a stable level of Bcl-xl and MDR1 whereas the *Bcl-2* silencing, so they did not attribute to the enhanced chemosensitivity of *Bcl-2*-silenced cells to DXR.

In accordance with its growth-promoting regulatory role, cyclin D1 could perform multiple functions as an oncogene, by enhancing several processes during malignant cell transformation, including accelerated cell proliferation, angiogenesis and resistance to apoptosis. Cyclin D1 also contributes to cell proliferation in osteosarcoma. For example, cyclin D1 downregulation by the 1-methyl-4-phenylpyridinium ion (MPP+) induces cell cycle arrest in p53-deficient MG63 osteosarcoma cells [25]. Transient DXR treatment downregulates the NF-kappaB-regulated gene Bcl-2, cyclin D1, thus potentiating apoptosis [26]. However, chronic exposure to DXR induced an obvious upregulation of cyclin D1 in human osteosarcoma MG63 cells, as shown in our study. Furthermore, our study demonstrated that silencing *Bcl-2* is associated with deregulation of cyclin D1. A possible mechanism is that Bcl-2 prevents apoptosis induced by loss of cell adhesion, thus induces cyclin D1 expression through the activated focal adhesion kinase (FAK) pathway [13]. However, the precise mechanisms of cyclin D1 upregulation by Bcl-2 remain to be determined.

An increased Bax/Bcl-2 ratio is associated with inhibition of proliferation in breast tumors [27]. Similarly, we found that cell proliferation was also suppressed in *Bcl-2*-silenced MG63/DXR cells. We demonstrated that the decreased proliferation depends on the increased apoptosis induced by combination of doxorubicin and *Bcl-2* siRNA, as well as the cell cycle arrest by downregulation of cyclin D1 in the *Bcl-2* siRNA-infected MG63/DXR cells.

In conclusion, our work provides evidence that downregulation of *Bcl-2* gene expression using lentivirally-delivered siRNA enhances the sensitivity to chemotherapy of osteosarcoma cells *in vitro*. Silencing of *Bcl-2* in MG63 cells is also associated with elevated Bax and decreased cyclin D1 levels; however, the molecular mechanisms for this are not yet known. Lentiviral RNAi targeting of *Bcl-2* could be a potential therapeutic strategy for osteosarcoma.

Acknowledgments

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